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## **LYSOZYME-MEDIATED FORMATION OF PROTEIN-SILICA NANO-COMPOSITES FOR BIOSENSING APPLICATIONS (POSTPRINT)**

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Madhumati Ramanathan and Aleksandr L. Simonian  
Materials Research and Education Center  
Samuel Ginn College of Engineering  
Auburn University  
Auburn, AL 36849

Heather R. Luckarift  
Universal Technology Corporation  
1270 North Fairfield Road  
Dayton, OH 45432

Ainur Sarsenova and Erlan Ramanculov  
National Center of Biotechnology of the Republic of Kazakhstan]  
Valikhanov Street 43  
Astana 010000, Kazakhstan

James R. Wild  
Biochemistry and Biophysics Department  
Texas A&M University  
College Station, TX 77843

Eric V. Olsen  
Clinical Research Facility  
81<sup>st</sup> Medical Group  
Keesler Air Force Base, MS 39534

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^National Center of Biotechnology of the Republic of Kazakhstan, Valikhanov Street 43, Astana 010000, Kazakhstan;

^^Biochemistry and Biophysics Department, Texas A&M University, College Station, TX 77843;

#Clinical Research Facility, 81st Medical Group, Keesler Air Force Base, MS 39534



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## Lysozyme-mediated formation of protein–silica nano-composites for biosensing applications

Madhumati Ramanathan<sup>a</sup>, Heather R. Luckarift<sup>b,c</sup>, Ainur Sarsenova<sup>d</sup>, James R. Wild<sup>e</sup>, Erlan K. Ramanculov<sup>d</sup>, Eric V. Olsen<sup>f</sup>, Aleksandr L. Simonian<sup>a,\*</sup><sup>a</sup> Materials Research and Education Center, Samuel Ginn College of Engineering, Auburn University, Auburn, AL 36849, USA<sup>b</sup> Air Force Research Laboratory, 139 Barnes Drive, Suite # 2, Tyndall AFB, FL 32403, USA<sup>c</sup> Universal Technology Corporation, 1270 N. Fairfield Road, Dayton, OH 45432, USA<sup>d</sup> National Center of Biotechnology of the Republic of Kazakhstan, Valikhanov Street 43, Astana 010000, Kazakhstan<sup>e</sup> Biochemistry and Biophysics Department, Texas A&M University, College Station, TX 77843, USA<sup>f</sup> Clinical Research Facility, 81st Medical Group, Keesler AFB, MS 39534, USA

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## ABSTRACT

We demonstrate a rapid method for enzyme immobilization directly on a waveguide surface by encapsulation in a silica matrix. Organophosphate hydrolase (OPH), an enzyme that catalytically hydrolyzes organophosphates, was used as a model enzyme to demonstrate the utility of lysozyme-mediated silica formation for enzyme stabilization. Silica morphology and the efficiency of OPH encapsulation were directly influenced by the precursor choice used in silica formation. Covalent attachment of the lysozyme template directly to the waveguide surface provided a stable basis for silica formation and significantly increased the surface area for OPH encapsulation. OPH conjugated to a pH-responsive fluorophore was encapsulated in silica and patterned to a waveguide surface to demonstrate the immobilization strategy for the development of an organophosphate array biodetector. Silica-encapsulated OPH retained its catalytic activity for nearly 60 days with a detection limit of paraoxon of  $\sim 35 \mu\text{M}$ . The encapsulation technique provides a potentially versatile tool with specific application to biosensor development.

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## 1. Introduction

The *in situ* encapsulation of biomolecules directly at a waveguide surface provides a potentially versatile tool with specific application to biosensor development. Various surfaces coated with titania nanoparticles, for example, have found increasing application in fields as diverse as photocatalysis, photovoltaics, photoelectrochromics and sensors due to the high photocatalytic activities of titania coatings [1–4]. Enzymes have been immobilized onto titania-coated waveguides via physical adsorption and covalent immobilization for biosensing applications and these techniques have been shown to enhance protein loading and retention of biomolecular activity [5,6]. Apart from photocatalytic activity, interesting optical properties of titania nanoparticles, i.e. high refractive index and dielectric constants have enabled their use as waveguides when coated on glass slides and optical fibers [7–9]. Interfacing additional inorganic oxides, such as silica, in layers or core–shell configurations are also known to tailor photocatalytic

properties and enhance stability [10]. In addition, studies show that specific surface modifications of titania coatings provides appreciable biocompatibility [11].

For biosensor applications, however, complications arise when enzymes are directly introduced into oxide structures due to the extreme conditions required for inorganic oxide synthesis that are unfavorable for retention of activity in biomolecules. Recent research in biomineralization reactions, however, offers an alternative paradigm for the formation of silica nanoparticles under mild conditions that provides stability and retention of activity of the encapsulated biomolecules [12]. Studies on mineralization of silica in biological systems, for example, have led to the isolation of polycationic species that catalyze silica formation including silaffins and silicatein proteins, from marine diatoms and sponges respectively [13–15]. Recently the lysozyme-mediated formation of silica particles has also been demonstrated [16,17]. Lysozyme as a template for biomineralization is advantageous as it is a ubiquitous protein and commercially available as opposed to specific silicification proteins that are expensive and labor intensive to purify. The resulting bio-nano-composites of lysozyme with amorphous silica have been shown to retain the proteins native anti-bacterial activity [17]. Aside from being a benign method for oxide synthesis, the method also provides an effective method for enzyme immobilization that

\* Corresponding author at: 274 Wilmore Laboratories, Auburn University, Auburn, AL 36849, USA. Tel.: +1 334 844 4485; fax: +1 334 844 3400.

E-mail address: [simonan@auburn.edu](mailto:simonan@auburn.edu) (A.L. Simonian).

is amenable to encapsulation and stabilization of enzymes at a surface. For example, lysozyme-mediated silica formation was successfully utilized to encapsulate organophosphate hydrolase (OPH) on a gold surface plasmon resonance waveguide [18]. Similarly, a silica-precipitating peptide, mediated silica formation and encapsulation of  $\beta$ -galactosidase *in situ* on functionalized silicon wafers [19]. In both cases, the encapsulated enzymes retained catalytic activity detectable by their respective analytes, namely paraoxon (for OPH) and lactose (for  $\beta$ -galactosidase) and provides a basis for biosensing applications.

In this study, we extended the versatile mineralization reactions of lysozyme to coat silica particles on waveguides formed from glass slides, pretreated with a titanium dioxide coating. The choice of precursor and immobilization chemistry were studied in order to optimize encapsulation of OPH as a model enzyme. The stabilized OPH composites were patterned to form an array of sensors on the waveguide surface and tested on a commercially available array biosensor to demonstrate the versatility of the enzyme encapsulation methodology for biosensor development.

## 2. Experimental

### 2.1. Chemicals and reagents

Lysozyme (from hen egg white), tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS) were purchased from Sigma–Aldrich (St. Louis, MO). 3-Mercaptopropyl trimethoxy silane (MPTS) was purchased from Gelest Inc. (Morrisville, PA). Potassium phosphate buffer (0.1N NaOH, 0.1 M  $\text{KH}_2\text{PO}_4$ , pH 8.2), CHES buffer for spectrophotometer assays (0.05 M CHES, 0.05 mM  $\text{CoCl}_2$ , pH 9.0), CHES buffer for the array biosensor (0.01 M CHES, 0.05 mM  $\text{CoCl}_2$ , 2.7 mM KCl, 120 mM NaCl, pH 8.5) and phosphate buffered saline (10 mM, pH 8.1) were prepared using reagents from stan-

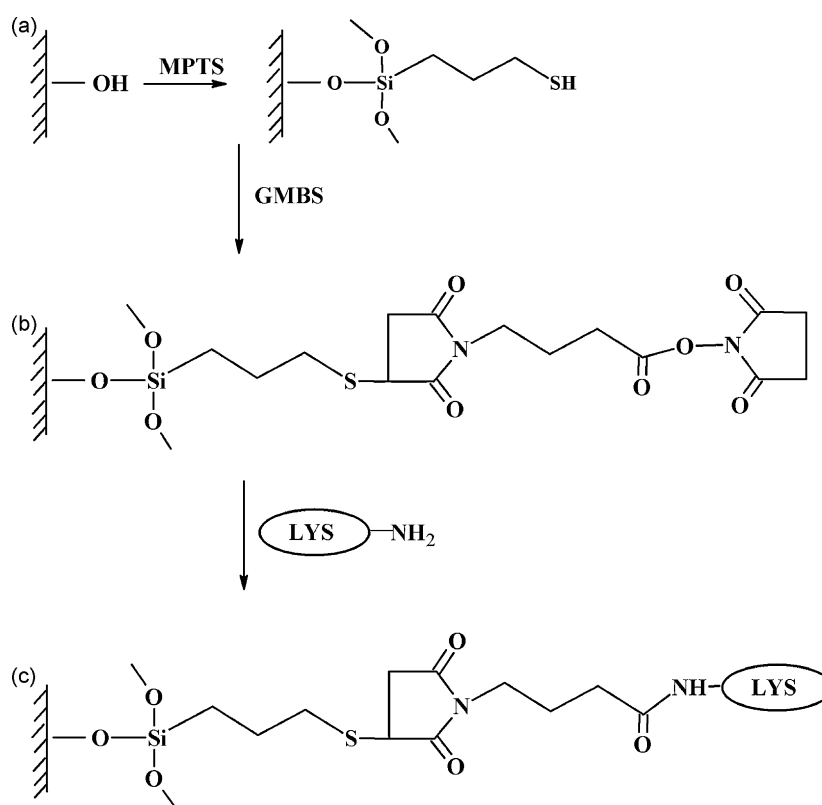
dard commercial sources. Hydrochloric acid, acetone, bovine serum albumin (BSA, fract V, cold alcohol precipitated) and dimethyl sulfoxide (DMSO) were all purchased from Fisher Scientific (Pittsburgh, PA). Titanium oxide nanopaste “T” was purchased from Solaronix Inc. (Aubonne, Switzerland) and plain pre-cleaned glass slides were purchased from VWR Chemicals (West Chester, PA). The solvents, absolute ethanol (Florida Distillers Co.) and anhydrous drisolv toluene were purchased from EMD Biosciences Inc. (Gibbstown, NJ). *N*- $\gamma$ -maleimidobutyryloxy succinimide ester (GMBS) was purchased from Pierce (Rockford, IL) and CNF (5-(and-6-)carboxynaphthofluorescein-succinimidyl ester, mixed isomers) was purchased from Invitrogen (Carlsbad, CA). Wild-type OPH (E.C. 3.1.8.1) from recombinant *Escherichia coli* was purified at Texas A&M as described elsewhere [20]. Paraoxon (diethyl-*p*-nitro phenyl phosphate) was purchased from Chem. Service (West Chester, PA). DI (de-ionized water, 18.2 M $\Omega$  cm) was used throughout and was obtained using a Millipore water purification system.

### 2.2. Instrumentation

A standard UV/vis spectrophotometer (Ultraspec™ 2100 pro, Amersham Biosciences, Piscataway, NJ) was used for all absorbance-based measurements. An Array Biosensor portable unit from the Naval Research Laboratories (Washington, DC) was used for all fluorescence-based assays with modifications as described previously [5].

### 2.3. Preparation of titanium oxide coated glass slides

A glass microscope slide forms the basis of the biosensor array and was pretreated with an initial coating of titanium oxide nanoparticles in a manner described previously [5]. Briefly, plain glass slides were cleaned in hydrochloric acid (12.1N) and thor-



**Fig. 1.** Schematic representation of the chemistry used for lysozyme immobilization. (a) Precoated slide with mercapto silane functionalization, (b) silanized slide with GMBS crosslinker and (c) immobilization of lysozyme.

oroughly rinsed in DI water before exposure to air plasma. After plasma cleaning, the slides were coated with titanium nanoxide paste (T series, Solaronix SA, Switzerland) using the doctor blade squeegee printing method as recommended by the manufacturer. The coated slides were air-dried, followed by a sintering treatment at 450 °C for 30 min. After cooling to room temperature, the slides were cut (1.5 cm × 1 cm) and cleaned in HCl (1N for 20 min) followed by a DI water rinse. The slides were then dried under a nitrogen atmosphere and used for chemical functionalization. To differentiate between the initial titania paste coating and subsequent modifications, the titania-coated glass slides as prepared above will be referred to simply as 'pretreated glass slides'.

#### 2.4. Functionalization of pretreated glass slides for lysozyme immobilization

The pretreated glass slides were chemically functionalized with MPTS and GMBS crosslinker in order to covalently immobilize lysozyme. Pretreated glass slides were treated with 2% MPTS (in anhydrous toluene) for 45 min, and then rinsed in toluene. Silanated slides, after rinsing, were dried and incubated with 10 mM GMBS (prepared in absolute ethanol, diluted from a stock solution prepared in 10% DMSO, v/v) for 45 min. GMBS-treated slides were rinsed sequentially in ethanol and water and incubated with lysozyme (5 mg/ml in 10 mM PBS, pH 8.1) for 5 h. Fig. 1 is a schematic representation of the chemistry used for lysozyme immobilization. In a control experiment, physical adsorption was used to immobilize lysozyme on pretreated glass slides by incubating with lysozyme (5 mg/ml in 10 mM PBS, pH 8.1) for 5 h before rinsing with water as before.

#### 2.5. Formation of OPH-encapsulated silica particles on pretreated glass slides

Two precursors were investigated for silica formation: in the first method, TMOS (30  $\mu$ l hydrolyzed with 170  $\mu$ l of 1 mM HCl) was mixed with 820  $\mu$ l potassium phosphate buffer (of 0.1 M, pH 8.1) containing 100  $\mu$ l of OPH (~1.5 mg/ml). A similar solution was prepared using TEOS in place of TMOS. 10  $\mu$ l of the above reaction mixtures were spotted on the pretreated glass slides and incubated for 5 h at room temperature.

For fluorescence-based assays, immobilization of lysozyme was carried out using the MPTS–GMBS functionalization described in the previous section. OPH and BSA were conjugated with the fluorophore, CNF, using a protocol provided by the manufacturer. A centrifugal filter unit with a 30 kDa molecular weight cut-off was used to separate the conjugated protein from any unreacted dye. BSA was used as a scaffold protein for CNF immobilization as a negative control with no paraoxon-hydrolyzing activity. The methodology for patterning slides with the OPH/BSA–CNF conjugate involved the use of capillary tubes as described in a previous study [5].

#### 2.6. Microscopic characterization of silica particles

Treated slides were sputtered with a thin layer of gold and visualized by scanning electron microscopy (SEM, JEOL, model 840) in order to study and characterize the silica particles formed. No gold coating was required for characterization using optical microscopy (Aetos Technologies Inc., AL).

#### 2.7. Enzyme assays for OPH activity

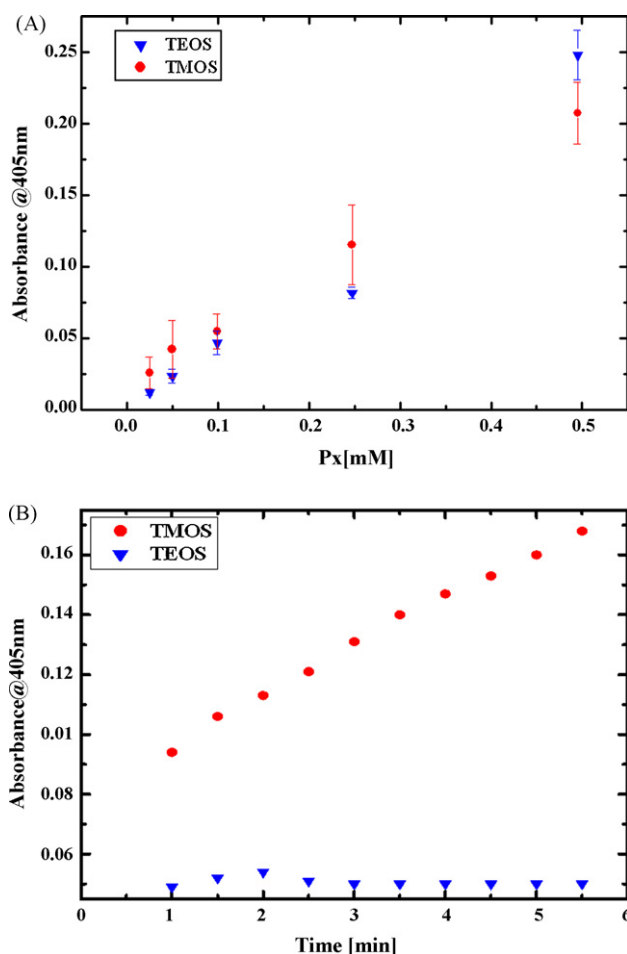
Activity of OPH was determined from the rate of catalytic hydrolysis of paraoxon to produce *p*-nitrophenol (PNP), which has a characteristic absorption peak at 405 nm. The conversion of

paraoxon was investigated at a range of paraoxon concentrations after incubation of OPH-immobilized slides for 1 min and measurement of the respective absorbance change at 405 nm.

#### 2.8. OPH activity assays on the array biosensor

Array biosensor assays were performed to test the efficiency of immobilization of OPH on the portable fluorimeter platform. Here, a red diode laser ( $\lambda = 635$  nm) directed over the edge of the glass slide undergoes total internal reflection producing evanescent waves that act as an excitation source (and direct measurement) for the labeled biomolecules immobilized onto the slide surface. A CCD camera was used to monitor the resulting fluorescent intensity.

The sensor was characterized for pH variation by passing 10 mM CHES buffer (at a pH ranging from 8.0 to 10.0) at a fixed flow rate of 1.5 ml/min for 30 s. The flow was stopped when the images were captured for data analysis. For paraoxon response, different concentrations of paraoxon were prepared in buffer (1 mM CHES, pH 8.4), and flowed through the channels of the sensor to pass through regions containing immobilized OPH and BSA and thereby form an array of sensing spots. Images containing the array of fluorescent spots were taken for exposure times of 3 s using Q capture pro software (Q Imaging, Canada). TIFF analyze software developed at NRL was used to quantify the fluorescent intensity of the captured images.



**Fig. 2.** (a) Hydrolysis of paraoxon to paranitrophenol by OPH encapsulated in silica using TMOS (●) and TEOS (▼) precursors and (b) leaching test: change in absorbance with time for a paraoxon solution after incubation with OPH-encapsulated silica slides using TMOS (●) and TEOS (▼) precursors.



### 3. Results and discussion

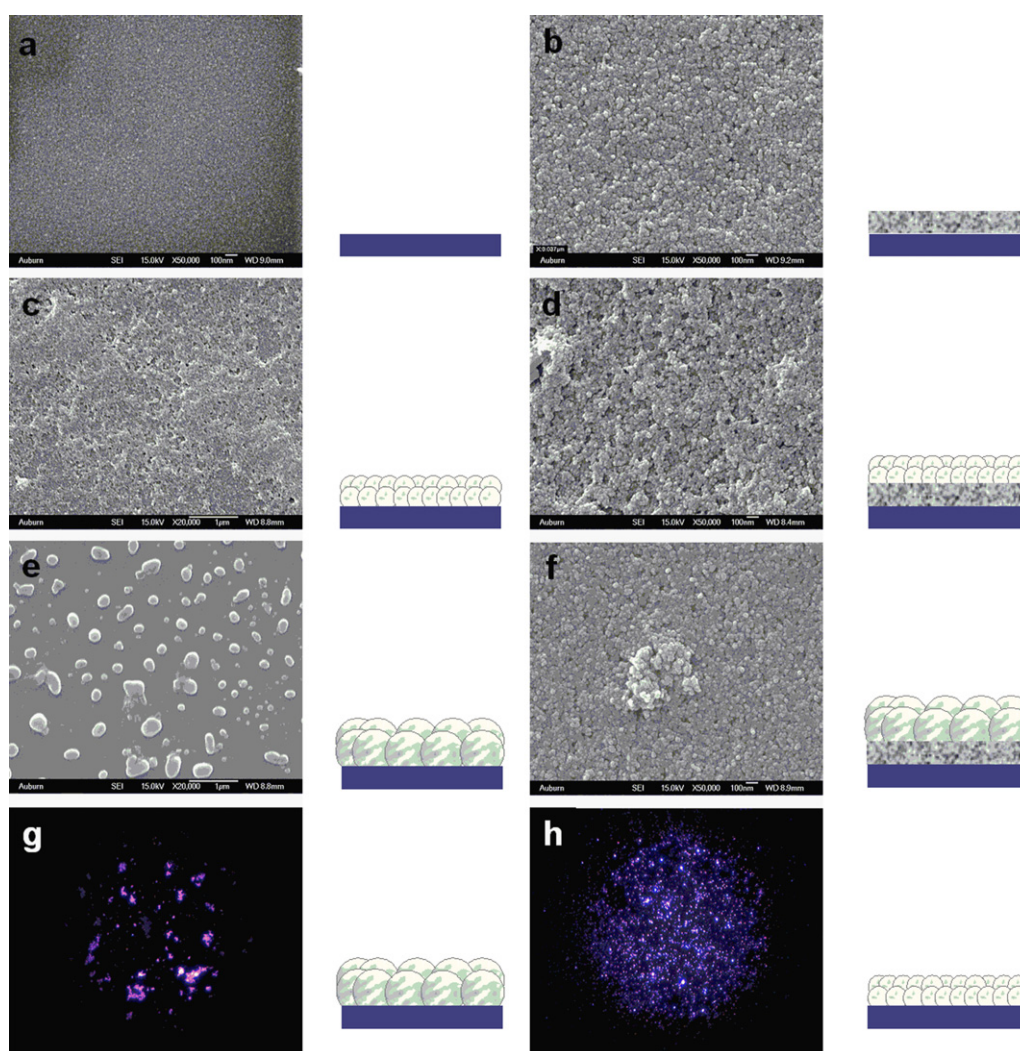
#### 3.1. Formation of silica particles for OPH encapsulation

Lysozyme catalyzes the formation of silica from its precursor solutions. Initially, the formation of silica particles and their ability to encapsulate OPH was investigated to ensure that the lysozyme-mediated formation of particles was not hindered when directed onto a pretreated surface. Lysozyme immobilization was achieved by physical adsorption and silica particles were formed *in situ*. OPH became encapsulated as the silica particles formed. The efficacy of the procedure was evaluated by monitoring the catalytic activity of encapsulated OPH for the hydrolysis of paraoxon. The graph in Fig. 2a shows the absorbance of PNP, obtained as a result of paraoxon hydrolysis by OPH encapsulated within silica composites formed directly on the pretreated glass slides. The response of OPH activity was comparable and the encapsulation efficiency showed little variation between the two silicate precursors investigated (TEOS and TMOS). Quantification of the amount of immobilized lysozyme template was determined by measuring the concentrations of lysozyme in solution before and after immobilization and in the subsequent wash steps. Under the reaction conditions described, approximately 30% of the initial lysozyme was immobilized on the pretreated glass slide by adsorption alone. Interestingly,

similar studies performed for OPH adsorption on the pretreated glass slides resulted in no retention of OPH and is attributed to poor electrostatic and/or hydrophobic interactions with the pretreated glass slide.

For silica encapsulation of OPH, TMOS as silicate precursor appeared to result in a silica matrix with increased sensitivity at low concentrations compared to TEOS but the standard error of measurements was more variable with TMOS as the precursor. It was considered that variability in the measurements may be due to OPH leaching from the silica particle matrix during analysis. In order to test this observation, the change in absorbance of the reaction solution was monitored after the functionalized slides had been removed from the reaction solution. After the removal of slides that contained OPH in silica prepared from TMOS, the hydrolysis of paraoxon continued, as evidenced by an increase in absorbance at 405 nm, indicating that OPH had leached from the surface of this preparation (Fig. 2b). In contrast, silica formation from TEOS produced a stable composite that prevented OPH from leaching over time.

The activity and retention of the encapsulated OPH may depend upon a number of factors, one of them being the morphology of particles formed during encapsulation. SEM analysis was used in order to characterize the matrix morphology and the effect of precursor on the resulting composites. The initial pretreatment of glass slides



**Fig. 3.** SEM and optical images of silica particles using TEOS and TMOS with schematics. SEM images of plain (a) and pretreated (b) slides, SEM images of silica particles formed using TMOS precursor on plain (c) and pretreated (d) slides, SEM images of silica particles formed using TEOS precursor on plain (e) and pretreated (f) slides. Optical microscopic images of OPH-encapsulated silica particles formed in solution mediated by lysozyme using TEOS (g) and TMOS (h) precursors.

with T-paste (titanium dioxide coating) results in a homogeneous coverage of nanoparticles that can be clearly differentiated from a non-coated glass slide. As such, encapsulated OPH was visualized for both pretreated and untreated glass slides. Fig. 3 shows that silica particles formed using TEOS appear pronounced and distinct with a wide particle size distribution (100–500 nm). This can be seen more clearly on plain glass slides than for pretreated glass slides due to the presence of the titania background coating. In the case of TMOS, the distribution of silica particles is more homogenous and uniform and produces a gel-like coating of silica particles. Optical images of the silica particles encapsulating OPH confirm that the TMOS precursor forms silica particles that are small and homogeneous with an even distribution of encapsulated enzyme, while the silica particles formed with the TEOS precursor again appear larger and possibly aggregated. This difference in particle morphology between the two silicate precursors requires further investigation as porosity of the matrix may contribute significantly to substrate interaction with the encapsulated enzyme and influence the stability of the matrix against leaching. In addition, preliminary observations suggest that the rate of silicification differs for the two precursors when catalyzed by lysozyme with silica formation from TEOS being a significantly more rapid process compared to TMOS (data not shown). The small particle distribution for TMOS–silica creates a large surface area that could explain the enhanced leaching of OPH as was observed in Fig. 2b. Thus, despite the heterogeneity of TEOS-based silica particles, the structure provides optimal retention for encapsulating OPH under the given experimental conditions. As such, for further experimental studies, TEOS was used as the precursor for silica formation.

### 3.2. Covalent attachment of lysozyme for silica formation

Preliminary studies confirmed that physical adsorption of lysozyme to pretreated glass slides did not hinder the formation of silica particles and OPH was successfully encapsulated in the resulting inorganic matrix. However, adsorption strategies of immobilization suffer from potential problems of protein leaching due to physiological changes in pH and ionic strength. For long-term operation of a biosensor, the stability of the matrix and corresponding retention of enzyme activity (of the sensing component) is paramount. In the study described herein, the possibility of lysozyme leaching from the preformed structure could in turn destabilize the encapsulated OPH immobilized on the surface. An alternative is to anchor the template lysozyme in place using covalent attachment chemistries to potentially enhance the stability and reusability of the final biosensor.

Covalent immobilization by MPTS modification allowed subsequent orientation of GMBS to preferentially bind the lysine residues of lysozyme. MPTS/GMBS-functionalized glass slides provided a platform for silica formation indicating that the orientation of lysozyme using covalent attachment did not significantly hinder the silicification reaction of lysozyme (Fig. 4). The protein immobilization efficiency indicated that ~90% of lysozyme was retained by MPTS/GMBS chemistry providing a significant increase to the loading capacity over physical adsorption alone (~30%). The efficiency of enzyme immobilization in silica was determined by measuring the activity of the encapsulated OPH. MPTS/GMBS-functionalized slides demonstrated linear detection rates (slope: 0.1945) corresponding to catalytic activity with increasing concentrations of paraoxon in the range 0.024–0.495 mM. The detection limit for the encapsulated OPH was approximately 0.05 mM paraoxon. Protein immobilization in which the immobilization matrix is covalently anchored to the pretreated glass slide should theoretically improve the long-term stability and hence applicability for bio-analytical devices. In addition, the use of a 3-dimensional matrix of silica significantly increases the surface area for protein loading [5,6]. In

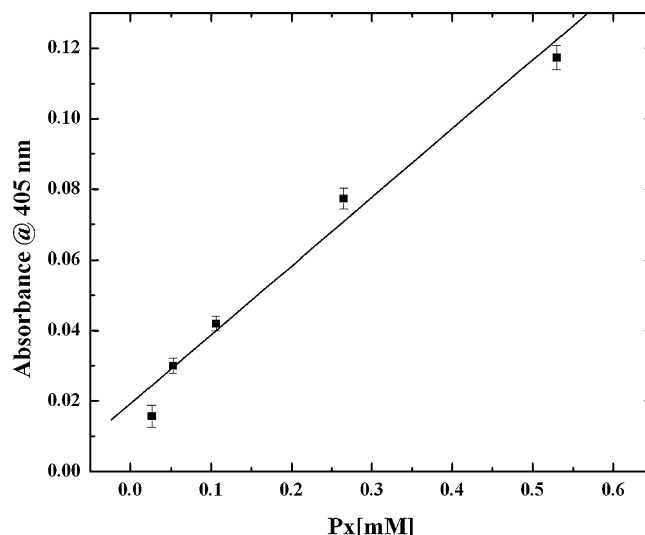


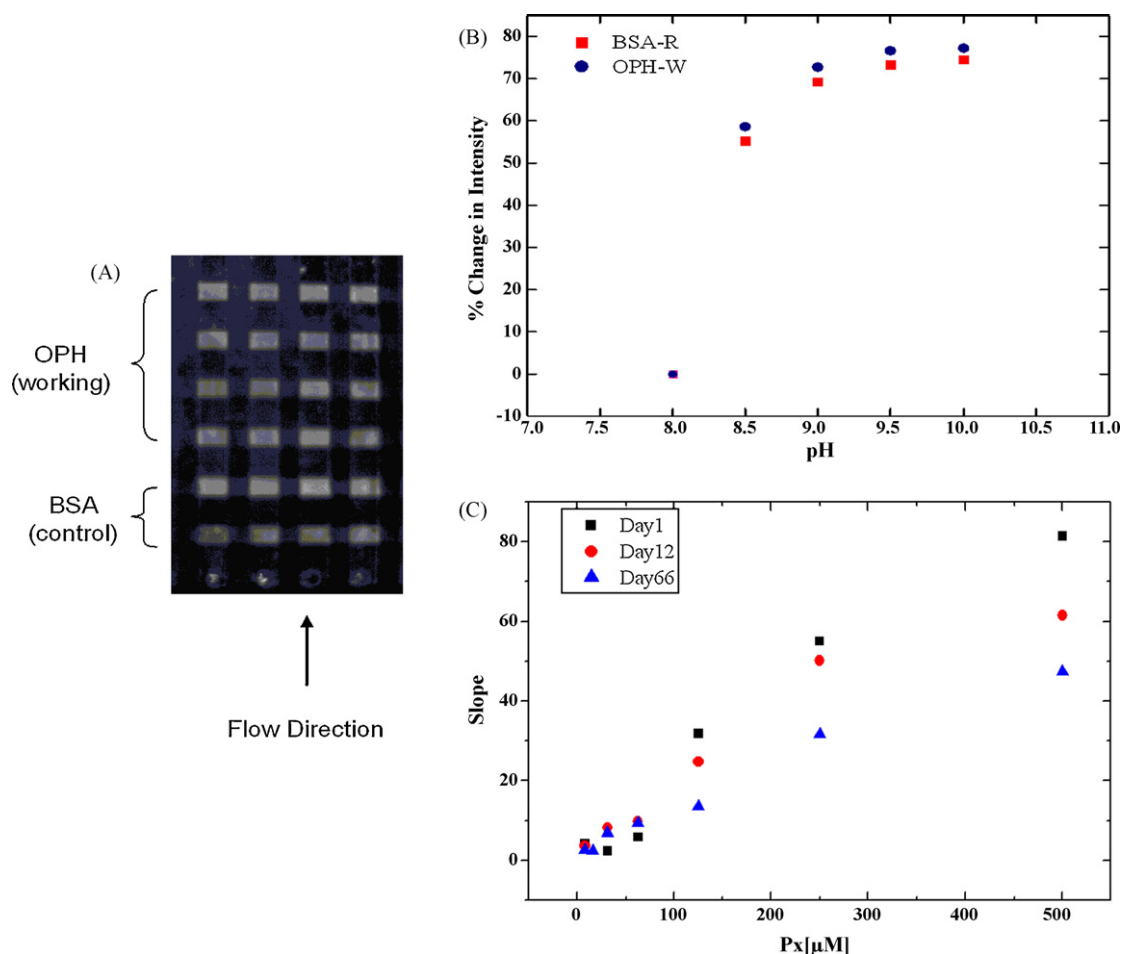
Fig. 4. Activity of OPH encapsulated in silica particles with covalent immobilization of lysozyme.

order to test the stability of the silica-encapsulated OPH, the retention of OPH activity was investigated following controlled changes in physiological conditions. High salt concentrations, for example, can be detrimental to protein stability in immobilized systems. The presence of 0.5 M sodium chloride in buffer solutions, for example, results in complete desorption of cytochrome C (a cationic protein with an isoelectric point (pI) similar to lysozyme, pI = 10 and 10.5 respectively) [21]. Pretreated glass slides were functionalized with MPTS/GMBS and lysozyme as described above and used to mediate the formation of silica containing OPH. The resulting functionalized slides were then treated with 0.5 M NaCl and retention of OPH activity was determined. No loss of OPH activity was observed following the high salt treatment, indicating that the silica particles provided stable encapsulation of OPH and offer protection against environmental changes. In contrast, a control slide in which OPH was attached in the absence of lysozyme-mediated silica resulted in a 33% loss in activity, due to the weaker binding of the OPH enzyme to the pretreated glass slide and the absence of the silica matrix to provide protection to the encapsulated enzyme. The retention of some portion of the activity in the absence of the silica matrix was attributed to the porous nature of the titania precoat used on the glass slide surface.

### 3.3. Encapsulation of fluorescent OPH for studies on an array biosensor

An array biosensor, developed at the Naval Research Laboratories was used to demonstrate the enzyme encapsulation described herein on a portable system. To directly integrate with the NRL array biosensor, the reporter for detection activity should be fluorescent-based and detectable using fluorescent dyes that excite at 635 nm and emit in the 665–680 nm range [22]. OPH was conjugated with the pH-dependent fluorophore, CNF, resulting in the utilization of only 50% of the actual fluorescent intensity of CNF due to the variations in available excitation wavelength (635 nm) from that required for CNF (598 nm) [5]. The catalytic hydrolysis of organophosphates by OPH generates protons which directly influences the fluorescence intensity of CNF. Mercaptosilane chemistry was used for lysozyme immobilization in order to pattern biomolecules for the biosensor array. MPTS/GMBS chemistry allowed for direct comparison with previous studies [5] that involved covalent immobilization of OPH. The pretreated glass slides were patterned with immobilized lysozyme and incubated





**Fig. 5.** Response of pretreated glass slides with patterned encapsulation of OPH–CNF on array biosensor. (a) Array of spots formed on glass slide and (b) pH response of working (OPH-W) and control (BSA-R) spots. (c) Paraoxon response of sensors over a period of nearly 2 months.

with a mixture of dye-conjugated OPH and silicate precursor solution. The lysozyme-mediated silica formation encapsulates the fluorescently labeled OPH directly at the sensor surface in a series of well-defined 'spots'. Control spots were similarly derived using fluorescently labeled BSA which allowed for direct variations in physiological conditions and environmental pH changes to be evaluated in order to eliminate external variations. Fig. 5a shows the patterned slide with 2 rows of control (BSA) spots and 4 rows of working (OPH) spots. The presence of the fluorescent spots indicated that the planar waveguide properties were not affected by a layer of silica at the surface. Fig. 5b shows the pH response of OPH conjugated to the pH sensitive fluorophore, CNF, encapsulated and patterned on the pretreated glass slide. BSA–CNF provides a control to differentiate between any changes in pH that may occur from hydrolysis of paraoxon and those due to sample-buffer variations. The graph shows a good correlation in pH response between the BSA and OPH spots, confirming that BSA was encapsulated in silica and was a suitable control for our assay (Fig. 5b).

Fig. 5c shows the paraoxon response, determined by calculating the slope for changes in fluorescent intensity over a period of time for specific concentrations of paraoxon. Though concentrations as low as 7 μM paraoxon could be detected, the statistical detection limit was ~35 μM. To determine the stability of the encapsulated enzymes, the measurements were repeated after 12 and 66 days of sensor preparation. The sensor retained more than 60% of its original activity even after 2 months of storage. By comparison, covalent attachment of OPH by MPTS/GMBS chemistry (to a similarly

pretreated glass slide) lost ~80% of activity within 18 days and 95% of OPH enzyme activity over a period of 59 days [5].

#### 4. Conclusions

In this study, we have demonstrated the utility of lysozyme to template silica nanoparticle formation on glass slides that have been precoated with a titanium dioxide paste. OPH was introduced during the synthesis procedure along with the ceramic precursors and the encapsulated OPH retained catalytic activity towards paraoxon. Under the given experimental conditions, TEOS as a precursor for silica formation was superior in encapsulating OPH than TMOS. Covalent attachment of lysozyme prior to silicification resulted in the formation of a stable silica matrix for OPH encapsulation with reproducible linear detection rates for paraoxon. Extension of the methodology to pattern silica and encapsulate OPH that was conjugated with a fluorescent indicator enabled the development of a spectro-fluorimetric array biosensor with OPH. OPH activity was retained within the resulting sensor array for over 60 days demonstrating a significant improvement in stability over previously reported configurations [5]. In addition, encapsulation in silica using this method is rapid with preparation times of a few hours, compared to the several days for covalent immobilization of OPH as reported previously [5]. OPH encapsulation was demonstrated herein as a model enzyme system, but the encapsulation methodology is applicable to a wide variety of biomolecules, providing the potential for multiple enzyme immobilizations and simultaneous analysis of multiple analytes [12].

The ability to integrate enzymes for catalytic detection with the capability to detect chemical agents may potentially extend the versatility of the array biosensor to both biological and chemical contaminants. The immobilization technique is rapid and potentially versatile for encapsulation of enzymes on the array biosensor surface using entrapment in silica particles. The only stipulation for integration is the presence of a fluorophore-based measurement that can integrate with the current systems 635 nm diode laser. Lysozyme-templated silica formation is readily adaptable to entrain fluorescently tagged enzymes and extend the current applicability of the array biosensor by providing a simple and facile method for encapsulation of biomolecules directly at the sensor surface.

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